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Original Article

Ototoxicity of styrene

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Abstract Styrene is extensively used in industry, but its ototoxicity, in particular in the pregnant female and the offspring, is still not well understood. In the current study, young adult male rats and pregnant female rats were exposed to styrene by gavage at different doses. The young adult male rats received a total of 12g/kg styrene within different periods (800 mg/kg/day for 5 days/week for 3 weeks, 400 mg/kg/day for 5 days/week for 6 weeks, 200 mg/kg/day for 5 days/week for 12 weeks, and 100 mg/kg/day for 5 days/week for 24 weeks) and the pregnant female rats received styrene at a dose of 400 mg/kg/day for 5 days/week for 6 weeks starting from the gestation day-4. Hearing loss and hair cell loss were assessed 5 days after the styrene treatment in the young adult male rats and in the mother rats. The cochlear impairments in the rat pups were examined 2 months after their birth. The styrene exposure caused hearing loss and hair cell loss starting from the mid-frequency region in the third row of outer hair cells (OHCs) and the impairments appeared to be related to the dosing level in each single day. Significantly, the styrene exposure to the pregnant rats interfered with auditory functional development of their fetus, leading to a deficit of cochlear amplification, although the OHCs appeared to develop well. The results indicate that a short-term high-level styrene exposure may be more ototoxic than a long-term low-level exposure for a similar total styrene dose and the styrene in the pregnant woman's body may interfere with auditory development of their fetus.

Key words Styrene ototoxicity; Pregnant rat; Exposure during gestation; Exposure during lactation

Introduction

Styrene is extensively used in industry in the production of plastics, fiberglass, synthetic rubbers, resins, insulators and protective surface coatings. [18, 39] Workers, including pregnant female workers, are at risk of exposure to styrene. In a recent measurement in a boatyard and a plastics factory, styrene concentrations ranged from 0.05 ppm (part per million) up to a maximum of 47 ppm [41]. However, individual exposure levels can be much higher than the averaged exposure level in the workplace. For example, in a report, styrene exposure levels among individuals ranged from 0 to 194 ppm with a mean level of 16.6 ppm .[30]

Occupational styrene exposure-related hearing loss has been observed in several investigations, [32, 35, 41] but not in other reports .[5, 31, 36, 40] However, it has to be pointed out that styrene-induced cochlear damage may occur without auditory functional loss. [14] To the best of our knowledge, styrene ototoxicity in the pregnant female and the offspring has never been studied.

Styrene ototoxicity in animals has been well documented. Styrene disrupts cochlear cells starting from the middle turn, leading to hearing loss in the mid-frequency region. [6, 12, 15, 17, 23-28, 46] Further studies have indicated that styrene targeted cochlear hair cells starting from OHCs in the third row, then to the second and first rows. [6, 12, 26] Inner hair cells (IHCs) are relatively insensitive to styrene exposure. Our recent study indicated that Deiters cells were more vulnerable than OHCs to styrene exposure and the styrene-induced

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cell death occurred through a caspase-dependent apoptotic pathway. Hensen cells played an important role in tissue healing after OHCs and Deiters cells were destroyed [12].

Studies of styrene toxicity in pregnant females showed that styrene exposure during gestation affected neonatal death rate, birth weight of the body and brain, neurotransmitters in the brain, and postnatal growth, although the alterations were not always observed [19-22]. This report indicates that styrene in the pregnant female's body may interfere with auditory functional development of the fetus.

Materials and Methods

Subjects

Thirty young adult male rats and eight pregnant female rats (Long Evans, 2.5 months of age) were acquired from Harlan Sprague Dawley Inc. (Indianapolis, IN). The pregnant female rats were acquired on day 3 of gestation and housed in the University at Buffalo animal facility. Twenty-four of the male rats and five of the eight pregnant rats were exposed to styrene. The remaining 6 male rats and 3 pregnant female rats were used as controls. The rat pups were born during gestational days 21-22. Seven of the eight litters had a normal size (10.9±1.9 pups per litter), but one of the styrene-treated litters had a small size (3 pups). A total of 51 rat pups were obtained and they were separated from their mothers after postnatal day 21.

All animal facilities are registered with the US Department of Agriculture and are inspected semiannually by the members of the Institutional Animal Care and Use Committee (IACUC) serving the Research Foundation of the State University of New York. Veterinarians were available at all times during the experiment. Background noise level in the colony room was 45 dB_A. Temperature was maintained at 22° C. Lights were on from 6:00am to 6:00pm. All procedures regarding the use and handling of animals and styrene exposures were reviewed and approved by the SUNY at Buffalo Institutional Animal Care and Use Committee.

To address whether the cochlear impairment is related to the total styrene exposure level or to the dose level applied in each day, the styrene exposure rats were divided into 4 groups and fed with the same total amount of styrene (12 g/kg), but at different daily dose levels. The rats in group 1 (n=6) were fed with styrene at a dose of 800 mg/kg/day for 5 days/week for 3 weeks (800-mg-exposure); the rats in group 2 (n=6) were fed with styrene at a dose of 400 mg/kg/day for 5 days/week for 6 weeks (400-mg-exposure); the rats in group 3 (n=6) were fed with styrene at a dose of 200 mg/kg/day for 5 days/week for 12 weeks (200-mg-exposure); and the rats in group 4 (n=6) were fed with styrene at a dose of 100 mg/kg/day for 5 days/week for 24 weeks (100-mg-exposure).

Styrene exposure

Styrene was mixed in olive oil (400 mg styrene in 800 ml oil). The male rats were exposed to styrene by gavage at a dose of 800 mg/kg/day for 5 days/week for 3 weeks (n=6), 400 mg/kg/day for 5 days/week for 6 weeks (n=6), 200 mg/kg/day for 5 days/week for 12 weeks (n=6), and 100 mg/kg/day for 5 days/week for 24 weeks (n=6). Five pregnant female rats were exposed to styrene starting from gestational day-4 by at a dose of 400 mg/kg/day for 5 days/week for 6 weeks.

Cochlear compound action potential (CAP)

Rats were anesthetized with ketamine (50 mg/kg, i.m.) and xylazine (6 mg/kg, i.m.). Body temperature was maintained at 37° C using a Homeothermic Blanket System (Harvard Apparatus). The round window of the right cochlea was surgically exposed and a silver wire electrode was carefully placed on the round window membrane for eliciting cochlear responses. A silver chloride reference electrode was placed in the neck muscles. Tone bursts (10-ms duration and 1-ms rise/fall time) at different frequencies (2, 6, 8, 12, 16, 20, 24, 30, and 40 kHz) were generated in a real time processor (TDT RP2.1, system-3, TDT, Gainesville, FL). The signals were passed through a TDT PA5 programmable attenuator and a power amplifier(HVA-1 HighVoltage amplifier), and then delivered to a high frequency earphone (using an ACO 1/2" microphone, 7013) placed within a speculum that opened to the ear drum. Sound levels at the position of the eardrum were calibrated for all the test frequencies. Cochlear potentials were amplified with a DAM50 Bio-amplifier (WPI). The gain of the preamplifier was 1000, and the

band of the filter was from 0.1 Hz to 3 kHz. Cochlear responses were averaged 50 times using a TDT RP2.1 real time processor and a custom written software using the MATLAB 6.1 software. CAP amplitudes were measured and plotted as a function of the stimulation level as a CAP input/output (I/O) function. CAP thresholds were defined as the stimulus level that elicited a CAP of 5mV in amplitude. Threshold shift in each animal was obtained by subtracting the mean CAP threshold in the control group from the measured animal.

Cochlear examination

Hair cell counting: Anesthetized animals were decapitated after cochlear functional measurement and the cochleae removed immediately. The round and oval windows and the apex of the cochlea were opened to facilitate perfusion. The cochlea was perfused with an incubation solution containing 0.05% nitrotetrazolium blue chloride (cat#N6876, Sigma), 0.05M sodium succinate, and 0.05M phosphate buffer and then incubated in the solution for 1 h (37° C). Nitrotetrazolium blue is an electron acceptor that, on reduction, precipitates as an insoluble and highly colored formazan. Succinate dehydrogenese (SDH) in the cell oxidizes sodium succinate and provides electrons for the reduction of the electron acceptor. Thus the cell is colored. The stained cochlea was fixed in 10% buffered formalin for 2 days. Cochlear micro-dissection was accomplished under a light microscope. Hair cells were counted under a light microscope (DMBA300 Digital Microscope, Microscope World) for producing cochleograms.

Examination of hair cell stereocilia and nuclei: Some cochleae were directly fixed in 10% buffered formalin. The organ of Corti was dissected out from the bony shell of the cochlea, and stained with FITC (fluorescein isothiocyanate) labeled phalloidin (Sigma) at a concentration of 5 μg / 1 ml phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) for 40 min at room temperature. The tissue was then co-labeled with PI (propidium iodide, Sigma) at a concentration of 5 mg / 1 ml PBS containing 0.5% BSA for 10 minutes. The stained specimens were mounted on slides with ProLong Gold antifade reagent (Invitrogen Molecular Probes) and examined using the Carl Zeiss Laser Scanning Systems LSM 510. Images were captured and analyzed using Zeiss LSM Image Examiner (v. 4.0.0.91).

Statistical analysis

Differences of audiograms and CAP I/O functions between groups were analyzed using two-way ANOVA. Differences at each individual point between groups were analyzed using t-test. A p-value < 0.05 was considered to be statistically significant.

Results

Figure 1 presents two images showing cochlear impairments after exposure to styrene at different dose levels. Styrene exposure at a dose of 400 mg/kg/day for 5 days/week for 3 weeks (6 g/kg) caused OHC loss mainly in the 3rd row and occasionally in the 2nd and 1st rows in the middle turn (Fig. 1A). Styrene exposure at a dose of 800 mg/kg/day for 5 days/week for 3 weeks (12 g/kg) caused almost a complete OHC loss and even some pillar cell loss (Fig. 1B, asterisks in the insert). Styrene o totoxicity appeared to be dose-dependent.

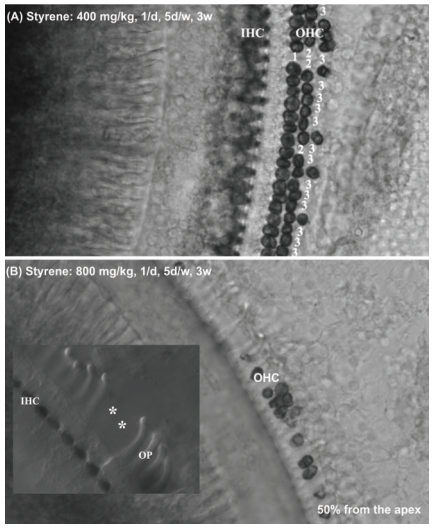

Fig. 1 Images showing cell loss in the cochlea after styrene exposure. (A) OHC loss starting from the third row after exposure to styrene at a dose of 400 mg/kg/day for 5 days/week for 3 weeks; (B) almost a complete loss of OHC and missing of some pillar cells (*) after exposure to styrene at a dose of 800 mg/kg/day for 5 days/week for 3 weeks.

Although the 4 groups of styrene exposed rats received the same total amount of styrene (12 g/kg), OHC loss (Fig. 2A) and CAP threshold shift (Fig. 2B) among the groups were significantly different ($p<0.0001$). The cochlear impairments appeared to be related to the

daily dose level, instead of the total amount of styrene. The 800–mg–exposure caused OHC loss of 50.8±5.1% (mean±SD) in the mid–turn in the cochlea (30–65%) and CAP threshold shift of 21.3 ± 9.4 dB in the frequency range of 8–24 kHz; the 400–mg–exposure caused OHC loss of 31.5 ± 6.0% and CAP threshold shift of 11.7 ± 4.4 dB; the 200–mg–exposure caused OHC loss of 5.4±3.0% and CAP threshold shift of 4.5± 3.6 dB; and the 100–mg–exposure caused OHC loss of 0.4±0.3% and CAP threshold shift of 4.3±3.4dB.

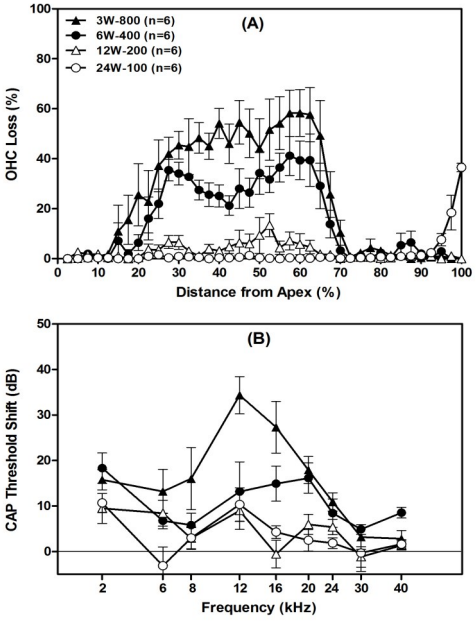

Fig. 2 OHC losses (A) and CAP threshold shifts (B) in young adult male rats after exposure to styrene of 12 g/kg with different dosing level per day (800 mg/kg/day, 400 mg/kg/day, 200 mg/kg/day, and 100 mg/kg/day). The impairments occurred in the mid–frequency region.

Styrene exposure at a dose of 400 mg/kg/day for 5 days/week for 6 weeks in the five pregnant female rats induced a CAP threshold shift of 10.3±4.7 dB in the frequency range of 8–24 kHz similar to that observed in the male rats (11.7±4.4 dB) (see open circles and triangles in Fig. 3). Significantly, styrene exposure in pregnant female rats resulted in hearing deficits in their offspring in the mid–frequency region consistent to that observed in their mothers. Two–way ANOVA analysis across stimulus frequency and t–test at each point did not find significant difference between the mother rats and the rat pups ($p>0.05$).

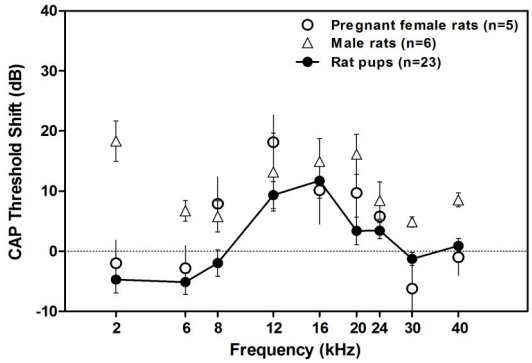

Fig. 3 CAP threshold shifts as a function of frequency in 5 pregnant female rats (open circles) their offspring (filled circles and solid line, n=23), and 6 male rats (open triangles). The pregnant female rats were exposed to styrene by gavage at a dose of 400 mg/kg/day for 5 days/week for 6 weeks starting from the gestational day 4. The male rats were exposed to styrene at a dose of 400 mg/kg/day for 5 days/week for 6 weeks. CAP thresholds were measured 5 days after the last day of the styrene exposure in the pregnant female rats and male rats and 2 months after birth in the rat pups. Vertical bars are standard error (SE).

The hearing deficits shown above in the rat pups were mainly from 2 (litter–2 and litter–3) of the 5 litters. In these 2 litters, the styrene exposure to the mother rats affected auditory sensitivity in both the mother rats and their offspring. Figure 4A presents CAP threshold shifts of individual rat pups from litter–3 (symbols) and their mean values (solid line). Styrene exposure caused a frequency–dependent threshold shift in the rat pups with a peak value of 27.1±2.7 dB at 16 kHz. The threshold shifts in the rat pups were similar to those observed in their mother (dashed line). Figure 4B presents CAP threshold shifts of individual rat pups from litter–2 (symbols) and their mean values (solid line). The styrene exposure also caused a frequency–dependent threshold shift in the rat pups in this litter with a slightly low peak value (15.5±4.0 dB) at 16 kHz.The mother rat also showed hearing loss, but at slight lower frequencies (dashed line). Figure 4C presents mean CAP input/output (I/O) functions at 16 kHz in the rat pups in litter–3 (filled circles), litter–2 (filled triangles), and control rats (open circles). The amplitudes of CAP in the pup rats of litter–2 (filled triangles) were similar to the control (open circles) at high stimulation levels, but lower than the control at low stimulation levels (<40 dB SPL),

indicating that styrene in the mother's body may interfere with the development of cochlear amplification (8, 12). The amplitudes of CAP in the pup rats of litter–3 (filled circles) were lower than the control at all stimulation levels, indicating that styrene in the mother's body may interfere with the development of not only cochlear amplification (OHCs) but also of the inner hair cell/spiral ganglion neurons. The differences between the pup rats and the controls at low stimulation levels (< 60 dB SPL) were significant.

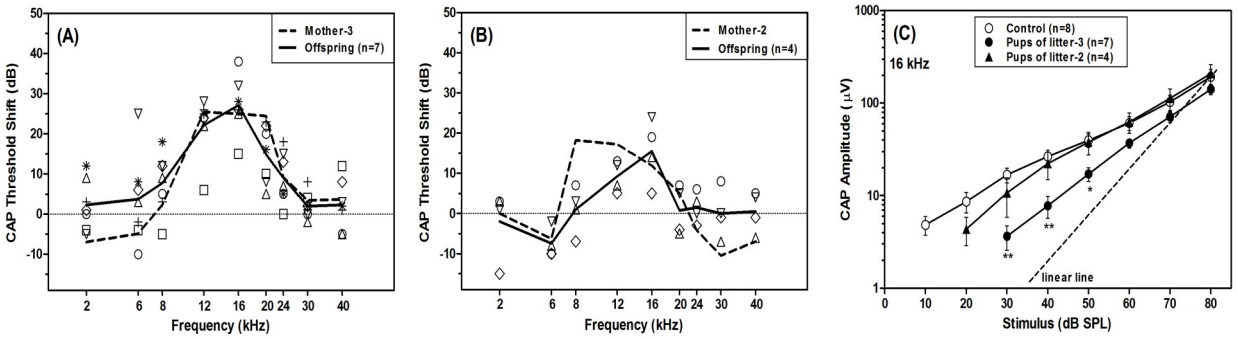

Fig. 4 CAP threshold shifts and amplitude reductions in 2 pregnant rats and their offspring showing severe styrene ototoxic effect on both the mother rats and their offspring. (A) CAP threshold shifts in the mother rat and the rat pups in litter–3; (B) CAP threshold shifts in the mother rat and the rat pups in litter–2; (C) CAP I/O functions at 16 kHz in the rat pups in litter–3 (filled circles), litter–2 (filled triangles), and controls (open circles). Symbols in (A) and (B) represent individual rat pups, and the solid lines represent the mean values. The dashed lines in (A) and (B) represent threshold shifts of the mother rats. The linear line in (C) represents a relationship of equal input and response, indicating a loss of cochlear amplification. The styrene exposure and CAP measurement were as described in Figure 3. *: $p<0.05$; **: $p<0.01$. Vertical bars are SE.

The mother rat of litter–1 showed CAP threshold shifts up to 28.6 dB after styrene exposure (see dashed line in Fig. 5A) and the mother rat of litter–5 also showed some hearing loss (17.3 dB at 12 kHz, Fig. 5B), but their offspring did not show hearing deficits (see symbols and the solid lines in Fig. 5A and B), indicating that styrene at an ototoxic level in the pregnant females interfere with auditory development of the fetus only in some cases. In litter–4, neither the mother rat nor its offspring showed hearing deficit (Fig. 5C).

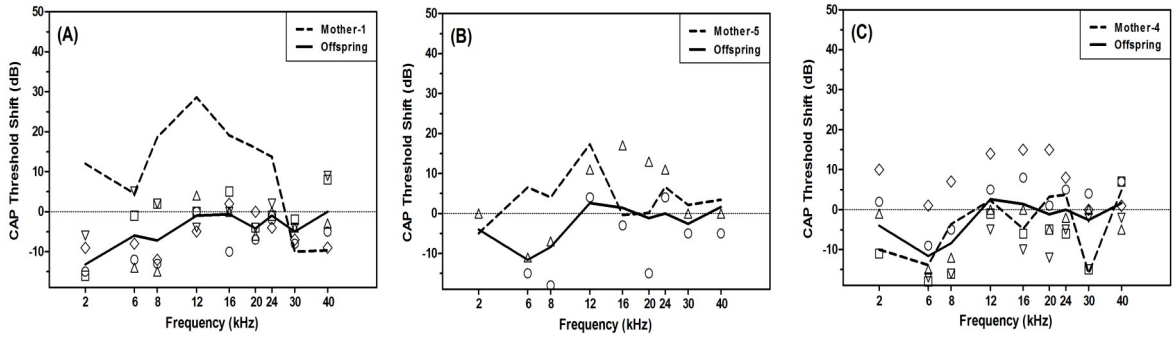

Fig. 5 CAP threshold shifts in the remaining 3 litters showing no styrene effect on the rat pups although styrene–induced hearing loss was observed in their mothers. (A and B): Hearing loss observed in the mother rats but no in their offspring; (C): No hearing loss observed in the mother rat and its offspring. Symbols represent individual rat pups. Solid lines represent mean threshold shifts in the rat pups and dashed lines represent threshold shifts in the mother rats. Styrene exposure and auditory threshold measurement were as described in Figure 3.

Styrene exposure caused a loss of about 1/3 of OHCs in the mid–turn (mainly in the third row) in all of the 5 pregnant female rats (Fig. 6, open circles). However, styrene in the mother's body did not significantly interfere with hair cell development (Fig. 6, filled circles).

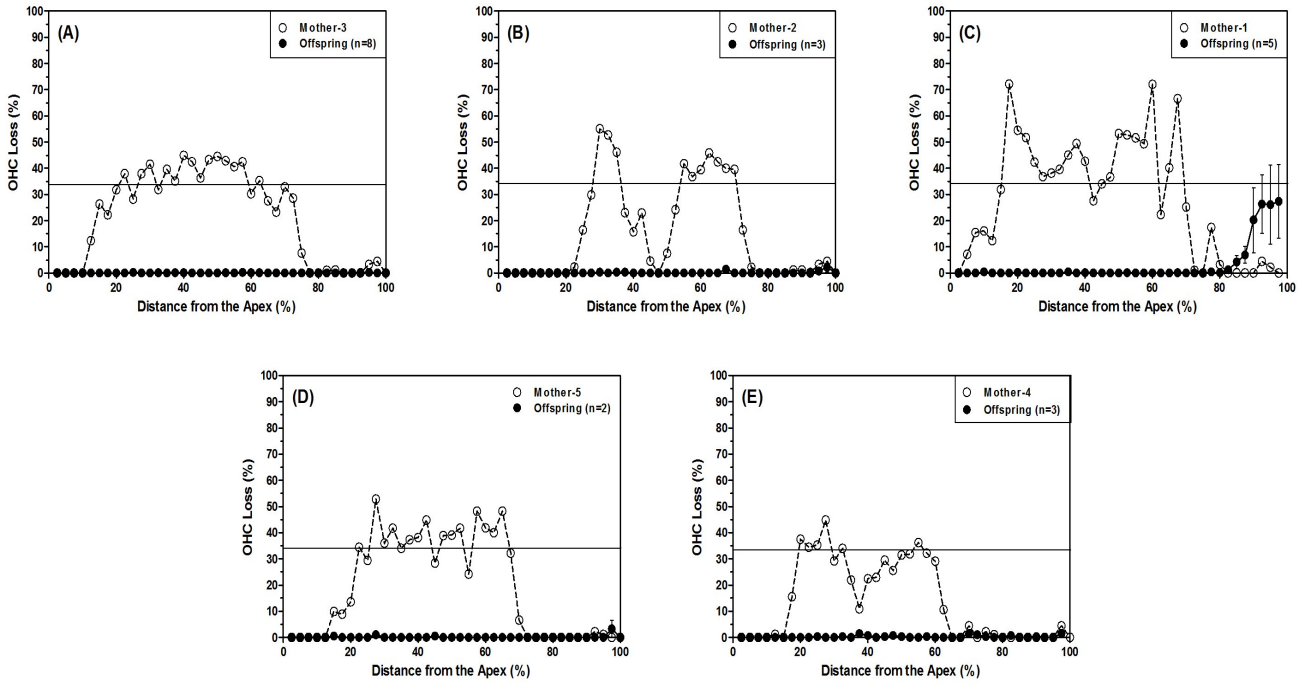

Fig. 6 OHC losses in the mother rats (open circles) and their offspring (filled circles) showing about one third of OHC loss in the mother rats but no OHC loss in the pup rats. Styrene exposure was as described in Figure 3. The cochleae were removed for examination after CAP recording. Vertical bars are

The rat pups in litter–3 showed a severe hearing deficit (see Fig. 4A), but without hair cell loss (see Fig. 6A). Four cochleae of the rat pups in litter–3 that showed severe hearing loss were examined. Although functional measurement suggested OHC injury, the OHCs looked intact. Figure 7 presents surface preparations showing OHC cuticular plates and stereocilia. The OHCs, from a region of 60% from the apex in a cochlea with a threshold shift of 23 dB at the related frequency (Fig. 7A), looked similar to the OHCs from a similar region in a control cochlea (Fig. 7B).

Figure 8 shows nuclei of OHCs, IHCs, Deiters cells, and Hensen cells from the middle cochlear region (52–56% from the apex). In two styrene–exposed cochleae (B and C), no distorted nuclei were seen compared to the control cochlea (A), although threshold shifts of 32 dB (B) and 26 dB (C) at the related frequencies were detected. Distorted nuclei of Hensen cells (condensation, marked with arrowheads) and OHCs (swelling, marked with asterisks) were observed in one styrene–exposed cochlea with a threshold shift of 26 dB at the related frequency (Fig 8D).

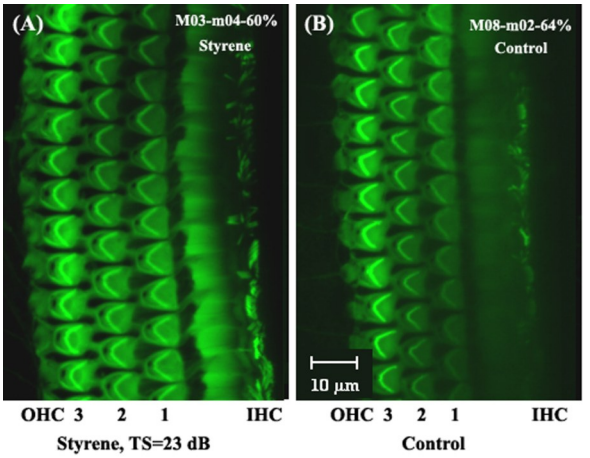

Fig. 7 Representative images showing OHC cuticular plates and stereocilia. (A) at a cochlear location of 60% from the apex in a rat pup in litter–3 with a CAP threshold shift of 23 dB at the related frequency (16 kHz); (B) at a cochlear location of 64% from the apex in a rat pup in litter–8 (control). Styrene exposure was as described in Figure 3. The horizontal bar represents 10 μm.

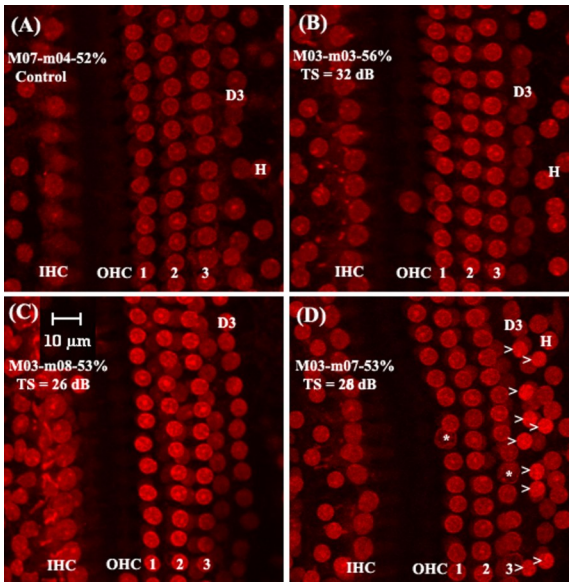

Fig. 8 Representative images showing nuclei of cochlear cells. (A) in a control rat pup at a cochlear location of 52% from the apex; (B) in a rat pup in litter-3 (M03-m03) at a location of 56% from the apex with a CAP threshold shift of 32 dB; (C) in a rat pup in litter-3 (M03-m08) at a location of 53% from the apex with a CAP threshold shift of 26 dB; (D) in a rat pup in litter-3 (M03-m07) at a location of 53% from the apex with a CAP threshold shift of 28 dB. D3: Deiters cells in the third row; IHC: inner hair cells; H: Hensen cells; OHC 1 2 3: outer hair cells in the first, second, and third row; arrowheads: condensed nuclei of Hensen cells; asterisks: swelling nuclei of OHCs. Styrene exposure was as described in Figure 3. The horizontal bar represents 10 mm.

Discussion

Styrene may cause hearing loss of mid-frequency. Styrene ototoxicity is strongly related to daily exposure levels, not the total exposure level. The pregnant female has a similar vulnerability to styrene exposure as the male. However, styrene in the pregnant female's body at the ototoxic level may interfere with auditory development of the fetus.

Styrene ototoxicity in pregnant rats

The styrene exposure (400 mg/kg/day for 5 days/week for 6 weeks) in this study caused about 1/3 of OHC loss in the mid-turn in the cochlea in pregnant female rats and about 10 dB of threshold shift in the mid-frequency region, which were similar to that observed in male rats. However, a few previous studies indicated more severe general toxiceffect of styrene in females. For example, a report showed that styrene blood levels after inhalation exposure were higher in female rats than that in male rats, [42] styrene exposure had a greater effect on body weight gain in female rats than in males [16]; and glutathione level in females were decreased after styrene inhalation to lower levels than in males.[34] In contrast, a report showed greater styrene toxicity in male mice than in female mice. [33] The ototoxic effect of styrene may not be related to the sex. It has to be noted that in one pregnant female rat (mother-4) styrene exposure caused OHC loss but not hearing loss. This is probably because the damage was limited in the third row. This phenomenon of styrene-induced OHC loss without hearing loss has been reported and discussed previously.[14] The surviving OHCs in some styrene-exposed cochlea with 33% of the OHCs missing may still be normally functioning and sufficient for maintaining the normal cochlear amp lification. [14]

Effect of styrene exposure to the pregnant female rats on their offspring

An important finding of this report is that styrene exposure to pregnant female rats during gestation and lactation interferes with auditory development of their offspring. The fetus can be exposed to styrene through the maternal blood. Styrene gavage at a dose of 400 mg/kg produces a blood level of about 10 mg/g in adult male rats.[13] This level can be higher in the mother rat because a previous study has showed higher blood styrene levels in female rats than in male rats after inhalation exposure. [42] Postnatal rat pups can be exposed to styrene through breast milk. Rat pups were separated from their mothers after postnatal day 21 in this study, before which the auditory system had been continuously developing. In the current study, styrene concentration in breast milk after styrene gavage was not measured. The styrene level in the milk should be higher than the blood styrene level because of the higher concentration of lipids in milk than in blood.

This study was designed to determine the maximal influence of styrene exposure to pregnant females on their offspring. More experiments are needed to show ototoxic effect of styrene in the mother's body on the baby when the mother is exposed to styrene during gestation only or lactation only.

Hearing loss was observed in many of the rat pups in the

middle frequency region, which is consistent with styrene ototoxic effect in the cochlea after direct exposure [6,12,15,17,24,25,26,26,27,28,46]. In spite of the hearing loss, all hair cells were present and looked intact in the cochlea in rat pups, except some in the basal 10% region in some rat pups. Only a few nuclei of OHCs and Hensen cells were abnormal in the middle turn in one rat pup with severe hearing loss. The data indicate that unlike in their mothers, styrene-related change in pup rats was not severe enough to cause death of hair cells, but the change wassufficient to interfere with the auditory functional development leading to a loss of cochlear amplification.

Hearing loss induced by intense noise has often been observed without hair cell loss in many experimental animals, including rats, mice, gerbils, guinea pigs, and rabbits, especially in the low to middle frequency region. [1-4,7-12,23,37,38,43-45] The hearing loss may be related to injury of hair cell stereocilia (4) or disruption of prestin, the OHC motor protein [8,29]. In this study, the cuticular plate and stereocilia of OHCs appeared to be intact in the cochlea of the rat pups with severe hearing loss (see Fig. 7). Styrene exposure during gestation may interfere with the production of prestin, the OHC motor protein, leading to a loss of OHC electromotility, and in turn a loss of cochlear amplification. More experiments are needed to find the change underlying the functional loss.

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Acknowledgments

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